Final Report

1. Title of Project:

Developing Aptamers to Methamphetamine as Nucleic Acid Sensors

2. Principal Investigator Names and Organization

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4. Abstract

Aptamers (small oligonucleotide sequences) are being selected that specifically recognize methamphetamine. The aptamers will be used for the development of a highly sensitive fluorescence-based assay. The work has progressed such that a population seems to be evolving that binds methamphetamine.

5. Project Description

The current project was to develop aptamers that recognize methamphetamine for the purpose of both detection and preventing methamphetamine effects. The reason for taking on this project was the possibility that we could provide a new reagent for combating the high level of methamphetamine abuse in the USA, which was described by the director of the NIDA as "an extremely serious and growing problem" [1]. Methamphetamine is a frequently used illicit drug in California and Hawaii; particularly in cities such as San Diego, San Francisco, and Honolulu. In San Diego, methamphetamine is currently the most frequently abused drug. Methamphetamine traffic has increased significantly in Arizona, Colorado, Iowa, Minnesota, Missouri, Nebraska and Washington. Methamphetamine abuse among teenagers is now common in the Midwest. An Department of Justice report states "an expert associated with Juvenile Court Services in Marshall County, Iowa, estimated in 1998 that one-third of the 1,600 students at Marshalltown High School had tried methamphetamine" [2]. Between 1994 and 1999 methamphetamine arrests rose almost 4-fold and methamphetamine lab seizures increased about 8-fold.

Two forms of amphetamine are Crank (a general term referring to a variety of forms of methamphetamine) and Ice (a crystalline methamphetamine that can be smoked). Methamphetamine effects are long lasting and continue to cause damage long after the user has stopped taking the drug. Long-term effects can include fatal kidney and lung disorders, possible brain damage, depression, hallucinations, disorganized lifestyle, permanent psychological problems, violent and aggressive behavior, weight loss, insomnia, behavior resembling paranoid schizophrenia, decreased social life, malnutrition, poor coping abilities, disturbance of personality development, lowered resistance to illnesses, liver damage, stroke, and death. Although the main effects are on the individual user, there are several significant effects of methamphetamine use on society. Societal impacts include the effects on others of car crashes, crimes, fires due to explosions from the illegal manufacture of methamphetamines, and hazardous waste. If methamphetamines are used during pregnancy, the resulting babies tend to be asocial, incapable of bonding, cry for 24 hours without stopping, and have tremors and/or birth defects.

Aptamers are small nucleic acids that avidly and selectively bind small ligands with affinities in the nanomolar to picomolar range and exquisite specificities [3]. For example, aptamers have been

isolated that distinguish caffeine from theophylline that differ by only a single methyl group [4] and that distinguish tyramine and dopamine that differ by a single hydroxyl group [5].

Many aptamers consist of a stem-loop structure in which the bases in the loop and the stem are intimately involved in the interaction with the ligand. These small nucleic acids can be selected by in vitro mutagenesis in a procedure called SELEX (systematic evolution of ligands by exponential enrichment) [6, 7]. DNA aptamers have been isolated to a variety of structures that include proteins and small molecules. Examples of ssDNA aptamers include aptamers that recognize thrombin, HIV reverse transcriptase, bile acids, cocaine, L-selectin, arginine, L-tyrosinamide and IgE [8-15]. The use of aptamers to analyze mind-altering drugs has already been demonstrated with a ssDNA aptamer that recognizes cocaine and that is the basis of a fluorescence quenching-based assay for analyzing cocaine [12].

Despite the suitability of aptamers to microarray formats, aptamer microarrays have not yet been developed and the microarray format for aptamers is itself a very new concept. For example, the term aptamer does not appear in the microarray glossary presented presented by the Cambridge Healthtech Institute as of March 2002 . The companies, Curagen and Somalogic have announced plans to develop microarrays of aptamers for detecting proteins in biological samples. But, to our knowledge, no plans have been announced for developing microarrays for detecting drugs or other small molecules.

6. Project Objectives:

The long-term goal of this project is to produce an aptamer microarray that can be used as a screening tool for a variety of drugs to provide forensics investigators with a rapid screening procedure for many common drugs. The following specific aims are proposed: 1) select for DNA aptamer(s) that recognizes methamphetamine 2) clone and characterize the isolated aptamer(s).

The aptamer could improve forensic science in crime laboratories by providing a new reagent for detecting methamphetamine in field samples where there is not direct evidence of methamphetamine synthesis. We anticipate that the aptamer(s) developed in this exploratory project will be directly applicable to the development of a homogeneous assay for methamphetamine that can be used to analyze samples for methamphetamine and other drugs. Aptamers can also be assembled as part of a microarray for analyzing series of drugs. Aptamer microarrays will have several advantages over immunoassays for the rapid screening of biological samples. Chief amongst these advantages are that: 1) The microarrays will be stable even when dehydrated and will be adaptable to mobile equipment, and 2) New aptamers can be rapidly "evolved" in the laboratory. Thus, the aptamer technology will be able to keep up with the introduction of new drugs and changes in drug use over time.

The aptamer(s) might also be applied towards decreasing a person’s response to methamphetamine. As a prototype for this type of approach, an aptamer that recognizes cocaine has been recently developed to be used to combat cocaine addiction [16].

7. Procedures

A methamphetamine-linked magnetic beads were used to select for the aptamer. First, the compound (Fig. 1) was prepared from the methyl ester of paraformylbenzoic acid and nitroethane and a primary amine in 78% yield (Kraus, unpublished). The crystalline product was then reduced in two steps to the amino ester which is hydrolyzed to the acid with lithium hydroxide (final yield , 49%). Methamphetamine-linked magnetic beads were used to select for the aptamer. First, the compound (Fig. 1) was prepared from the methyl ester of paraformylbenzoic acid and nitroethane and a primary amine in 78% yield (Kraus, unpublished). The crystalline product was then reduced in two steps to the amino ester which is hydrolyzed to the acid with lithium hydroxide (final yield , 49%). Dr. Kraus then employed the succinimidyl ester of biotin with the amine produced from the reaction of the resorcinol spacer with the chlorocarbonyl derivative of methamphetamine. The metamphetamine-2-nitro-(1,3 bis, amino-ethane)-resorcinol was then coupled to amine-reactive Dynabeads M-270 Epoxy according to the manufacturer's instructions to form the affinity resin.

We followed a procedure for making single stranded DNA (ssDNA) aptamers that has been previously described [6, 13, 17]. A mix of 2 nmole of randomly varied ssDNAs (1.4×10^{15} different sequences) was passed multiple times through a methamphetamine-agarose column with intervening PCR amplifications to introduce new variations into the sequence.

The methamphetamine-binding ssDNAs were removed from the affinity beads at each round by heating the beads and PCR was performed with the beads present. After each round, the pool size of the DNA in the mixture was monitored by determining the percent of total loaded ssDNA that was adsorbed and eluted from the affinity column. Negative selections against the beads with methamphetamine were also included.

8. Results/ Discussion

The project consisted of two parts. The first part of the project was the chemical synthesis of methamphetamine derivatives that were then used for the second part of the project, which was to select aptamers that specifically bound to methamphetamine.

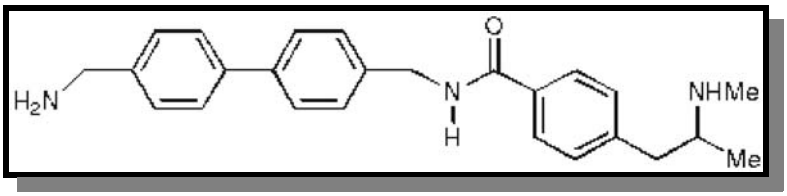

Figure 1. Resorcinol derivative of methampetamine.

Part one was successfully completed. First, the compound was prepared from the methyl ester of paraformylbenzoic acid and nitroethane and a primary amine in 78% yield (Kraus, unpublished). The crystalline product was then reduced in two steps to the amino ester which is hydrolyzed to the acid with lithium hydroxide (final yield, 49%). Dr. Kraus then employed the succinimidyl ester of biotin with the amine produced from the reaction of the resorcinol spacer with the chlorocarbonyl derivative of methamphetamine. The metamphetamine-2-nitro-(1,3 bis, amino-ethane)-resorcinol (Fig. 1) was then coupled to amine-reactive Dynabeads M-270 Epoxy according to the manufacturer's instructions to form the affinity resin.

Selection for a DNA aptamer was done according to the protocol outlined in Fig 2. Two sets of selections were performed. The first was taken through 13 rounds until it was determined that nothing was evolving from the original pool. The second selection was taken through 7 rounds and seems to be evolving an aptamer as shown in figure 3. The percent of the pool that bound the

methamphetamine on the affinity matrix increased dramatically on round 6. Because of this the conditions were made more stringent for the next round (7) and the number of washes of the matrix after binding the DNA were increased from one to 10. Even so the percent of the pool that bound the methamphetamine affinity matrix increased compared with round 5.

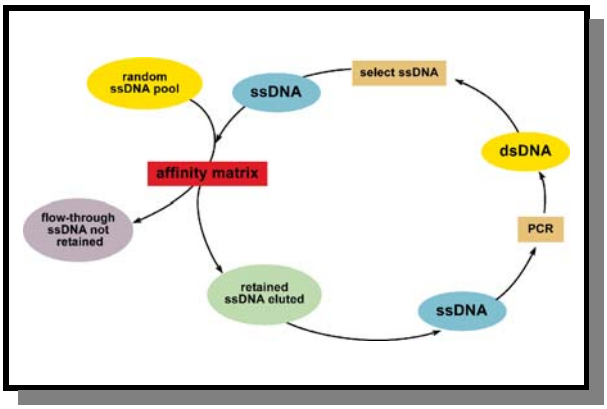

Figure 2. Selection protocol for a DNA aptamer. The figure demonstrates a single round of selection using an affinity matrix consisting of methamphetamine linked to magnetic beads by way of a resorcinol bridge.

9. Dissemination Discussion

Although the project has not reached completion, we have reasonably good evidence that an aptamer is evolving in our pool. The team is looking for opportunities for further funding to complete the project. Once an aptamer is selected, the results of the current proposed research will be disseminated by publication in research journals. The results of these studies will then be used to develop a microarray or other assay for methamphetamine and other drugs that can be used for the detection of these drugs at the crime scene and in the laboratory

10. Discussion of problems that have arisen.

When we started this project we were unaware of the amount of time that it would take to develop and optimize the aptamer selection. Therefore, we underestimated the amount of work that we could accomplish within the resources of the current grant. The better understanding of the true effort required for optimization of the protocols and aptamer selection that we have learned from these studies will help us to more appropriately define the necessary resources and time for completing this project. The results obtained from this project will contribute to the preliminary results used as a basis for obtaining further resources to complete the project.

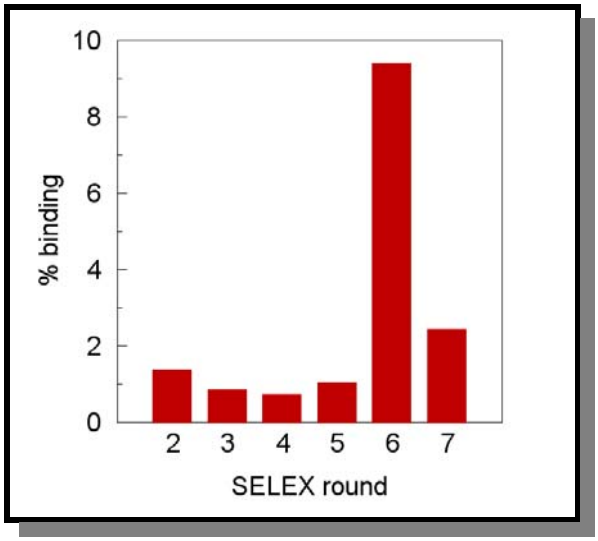

Figure 3. Selection of a methamphetamine aptamer. In this selection experiments seven rounds of selection were performed and the percent of the pool that bound to the column was determined at each round. These results are plotted in the figure.

11. Acknowledgement

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11. References

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